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Effect of Steroids on Gonadal Growth and Gametogenesis in the Juvenile Red Sea Urchin *Pseudocentrotus depressus*

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Abstract. Red sea urchins, 10 months old, were fed for 30 days on a casein-based diet containing progesterone, androstenedione, testosterone, estrone, or estradiol-17β. The mean gonad indices of male animals in the androstenedione- and the estrone-treated groups were significantly higher than those in the control group, suggesting that these steroids promote gonadal growth in male animals. Histological observations indicated that spermatogenesis in the estrone-treated group was also promoted compared to that in the control group. In contrast, female urchins were not obviously affected by the steroid-treated diets, probably because yearling female *P. depressus* are not otherwise ready to carry out gametogenesis. We conclude that androstenedione, estrone, and possibly their derivatives, are involved in the reproduction of male *P. depressus*.

Introduction

Sex-related steroids, which have regulatory functions in vertebrate reproduction, are also found in echinoderms. The roles and actions of steroids in starfish have been investigated often: *e.g.*, biosynthesis and metabolism (Schoenmakers, 1979; Schoenmakers and Voogt, 1980, 1981; Voogt and Van Rheenen, 1986; Voogt *et al.*, 1986, 1990, 1991a, b; Hines *et al.*, 1992a); seasonal variations in steroid levels (Schoenmakers and Dieleman, 1981; Voogt and Dieleman, 1984; Xu and Barker, 1990; Xu, 1991; Hines *et al.*, 1992b); and the effects of steroid injections (Schoenmakers *et al.*, 1981; Takahashi, 1982a, b; Barker and Xu, 1993). These previous reports suggest that steroids are involved in the reproduction of starfish.

The importance of steroids in sea urchin reproduction is less well known. Estradiol-17β induced the synthesis of a novel protein in the coelomocytes of *Dendraster excentricus* and *Strongylocentrotus purpuratus in vitro* (Harrington and Ozaki, 1986). Levels of estradiol-17β and progesterone were determined in the testes and ovaries of *Eucidaris tribuloides* every 3 months during the annual reproductive cycle (Hines *et al.*, 1992c). Oral administration of estrone increased the body weight of *Pseudocentrotus depressus*, the red sea urchin (Unuma *et al.*, 1996a). However, we are just beginning to understand the relationship between steroids and the reproduction of sea urchins.

In *P. depressus*, gonadal growth occurs from spring to late autumn, preceding and also during gametogenesis. This growth is mainly due to the accumulation of nutrients by the nutritive phagocytes that occupy the lumina of the gonads of both sexes. Gametogenesis normally begins in September or October, and gonads are filled with mature gametes in November or December (unpubl. data). To investigate the effect of steroids on gonadal growth and gametogenesis, we fed sex-related steroids to *P. depressus*. Casein-based diets containing progesterone, androstenedione, testosterone, estrone, or estradiol-17β were prepared and fed to juvenile red sea urchins for 30 days beginning in early September.

Materials and Methods

Animals

Individuals of *P. depressus* were hatched and reared at the Fukuoka Prefectural Fish Farming Center and were transferred to the Nansei Station of the National Research

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Table I

Composition of the experimental diets

Casein	30 g
Sodium alginate	30 g
Dextrin	20 g
Cellulose	5 g
Pollock viscera oil	5 g
Mineral mix[1]	4 g
Soybean lecithin	3 g
Vitamin mix[2]	2.3 g
Choline chloride	0.59 g
L(+)-Ascorbic acid	0.1 g
β-Carotene	0.01 g
Ethanol with steroid[3]	2 ml
Water	120 ml

[1] U. S. P. XII salt mixture with trace elements (Halver, 1957).

[2] Contains each vitamin corresponding to 44% of the premix reported by the National Research Council (1973).

[3] The dissolved steroid is one of the following: progesterone, androstenedione, testosterone, estrone, or estradiol-17β (2 m*M*). Control diet lacks steroid.

Institute of Aquaculture, Mie, Japan, in May 1993. These individuals, 6 months old, were kept in a 1000-liter tank supplied with sand-filtered seawater at 30 l · min^{-1} and were reared on kelp (*Eisenia bicyclis*) for 4 months until used in the feeding experiments. Before these experiments, 20 urchins were sacrificed and their gonads were examined. The average gonad index (GI) was 0.87%, and all the gonads were in Stage 0 as determined by histological observations. The stages of maturation are defined below.

Experimental diets

The experimental diets were formulated according to Akiyama *et al.* (1997) with some slight modifications (Table I). On a dry weight basis, 25% of the diet was protein from casein, the sole protein source. Five test diets, each containing a steroid, and a control diet that lacked steroid were tested. Progesterone, (+)-4-androstene-3,17-dione (androstenedione), testosterone, estrone, and estradiol-17β (Wako Pure Chemical Industries, Ltd., Tokyo) were dissolved in ethanol and added to the diet to make a concentration of 1.8×10^{-8} mol · g^{-1} wet weight of diet. Only ethanol was added to the control diet. The ingredients were mixed thoroughly, shaped into a cookie-like form (about 50 mm in diameter) and then soaked in 5% $CaCl_2$ solution for 10 min (Akiyama *et al.*, 1997). The prepared test diets were kept at −20°C until used.

Feeding procedure

The feeding experiments were conducted from 6 September to 6 October. Three hundred urchins, 20 mm in test diameter and 3.8 g in body weight, were divided into six experimental groups, each containing 50 urchins; five groups were each given one of the steroid diets, and one group was given the control diet. Each group was placed in two rectangular acrylic tanks (50 × 20 × 30 cm, holding 20 liter of water), with 25 individuals per tank. Sand-filtered seawater was supplied to the tanks at the rate of 0.7 l · min^{-1}. During the experimental period, the water temperature gradually decreased from 25°C to 21°C. Sea urchins in each tank were given an excess of the experimental diets (7–10 g per tank) every other day and were allowed to feed to satiation. Uneaten diets were collected and weighed before new diets were given. Food intake for each tank was estimated based on the decrease in weight of the diet. After 30 days of feeding, body weight and gonad weight were measured for all animals. Gonad index (GI) was calculated from the following formula.

GI (%) = 100 × gonad wet weight/body wet weight

Daily food consumption was calculated for each tank using the following formula.

Daily food consumption (%) = 100 × food intake/[(initial body weight + final body weight)/2] × rearing period (days)

Histological observations

Small pieces of gonad from each animal were fixed in Bouin's solution, embedded in paraffin, and sectioned at 10 μm. The sections were stained with hematoxylin and eosin, and then observed by light microscopy to determine the sex and gametogenic stage of the gonads. The gonadal maturity of each animal was assessed according to the six-stage classification of Fuji (1960), with some slight modifications (Unuma *et al.*, 1996b) as follows (see Fig. 1).

Stage 0 (neuter): No obvious germ cells are observed, and sexes are unidentifiable. The gonadal lumina are filled with nutritive phagocytes.

Stage 1 (developing virgin): A few small oocytes or small clusters of spermatogonia are present at the periphery of gonads otherwise filled with nutritive phagocytes.

Stage 2 (growing): The gonads contain rows of spermatogonia or of oocytes at the periphery. The center of the gonads still contain nutritive phagocytes.

Stage 3 (pre-mature): In the center of the gonad, nutritive phagocytes are replaced with spermatozoa or ripe ova.

Stage 4 (mature): The gonadal lumina are filled with ripe ova or spermatozoa. Nutritive phagocytes are recognized only at the periphery of gonads.

Stage 5 (spent): Gonadal lumina are almost empty, with a few relict ova or small masses of relict spermatozoa. Nutritive phagocytes are recognized at the periphery of gonads.

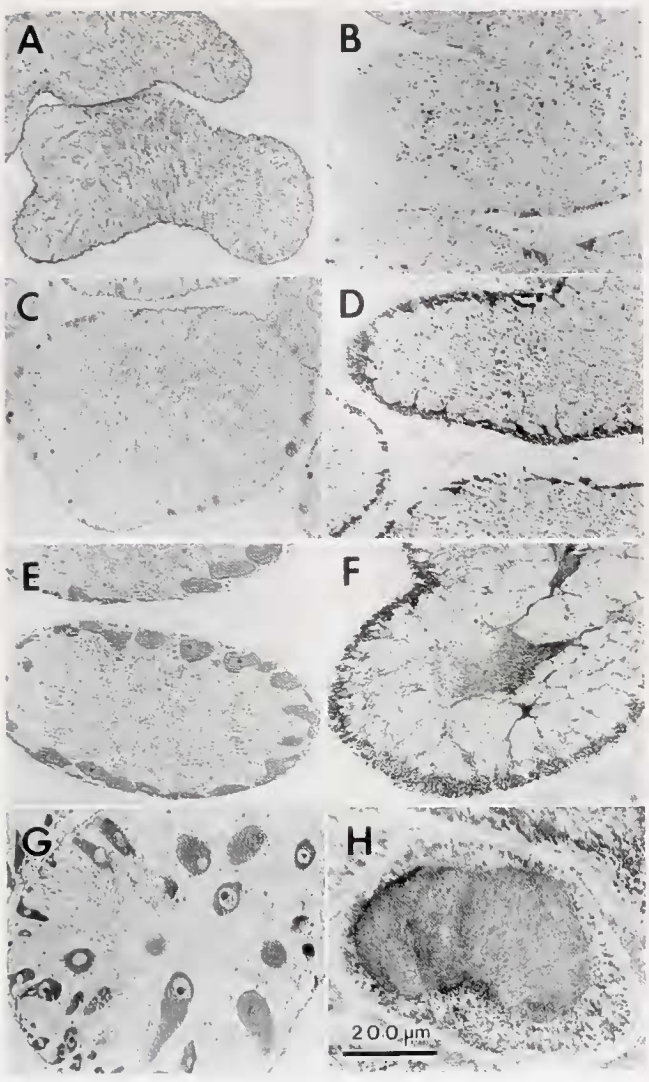

Figure 1. Classification of gonadal maturity in *Pseudocentrotus depressus*. Only the stages observed in this study are shown. B, D, F, H: male; C, E, G: female. (A) Stage 0: No obvious germ cells are observed, and sex is unidentifiable. (B, C) Stage 1: Small clusters of spermatogonia or a few young oocytes are present at the periphery of the gonad. (D, E) Stage 2: The gonads contain rows of spermatogonia or of oocytes. (F, G) Stage 3: In the center of the lumina, nutritive phagocytes are replaced with spermatozoa or ripe ova. (H) Stage 4: The gonadal lumina are filled with spermatozoa. Scale bar: 200 μm.

Statistical analysis

The mean values of GI and daily food consumption were compared using unpaired Student's t tests between the steroid-treated groups and the control group, after comparison of the variances using F test.

The Mann-Whitney U test was used to compare the distribution of the maturational stages of gonads between the steroid-fed groups and the control group.

Results

Gonad index

No mortality occurred during these experiments. Moreover, sex could be determined by histological observations in all but three animals. One specimen in each of the progesterone-, androstenedione-, and testosterone-treatment groups was neuter, and these three cases were omitted when average GIs were calculated for each sex.

Figure 2 shows the mean GI values for each sex after the 30-day feeding trial. In male animals, the mean GI of the control group was 4.87%. The androstenedione- and estrone-treated groups showed significantly higher values than the control group, 6.22% ($P < 0.05$) and 6.94% ($P < 0.001$), respectively. The values in the progesterone-, the testosterone-, and the estradiol-17β-treated groups were not significantly different from the control group.

Unlike the male animals, the female animals had similar mean GI values from all groups; in particular, no significant difference was found between the steroid-treated groups and the control group.

Maturational stages of gonads

Frequencies of the maturational stages of gonads for each sex are shown in Figure 3. In males, the percentages of Stages 1, 2, and 3 in the control group were 32%, 54% and 14%, respectively. In all the steroid-treated groups except that treated with estrone, the percentage of each stage was similar to that in the control group. In the estrone-treated group, however, the percentage of Stage 1 was only 4% (one-eighth that of the control group) and that of Stage 3 was 31% (more than twice that of the control group). The distribution of maturational stages in the estrone-treated group was significantly different ($P < 0.05$) from that of the

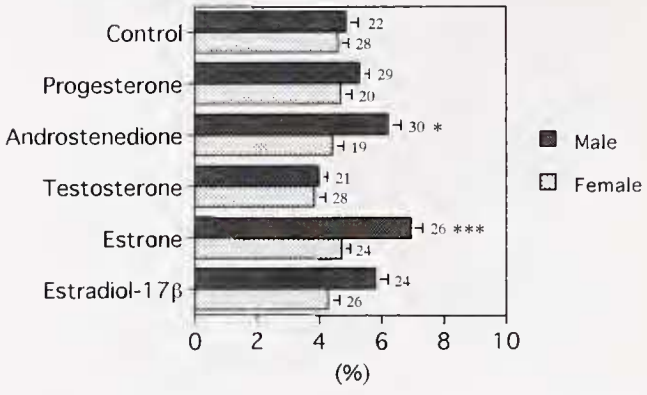

Figure 2. Gonad index of male and female *Pseudocentrotus depressus* fed diets containing steroids. Values represent the mean ± SE. Numerals in the graph indicate the number of individuals. Values with asterisks are significantly different from the control of the same sex (*, $P < 0.05$; ***, $P < 0.001$).

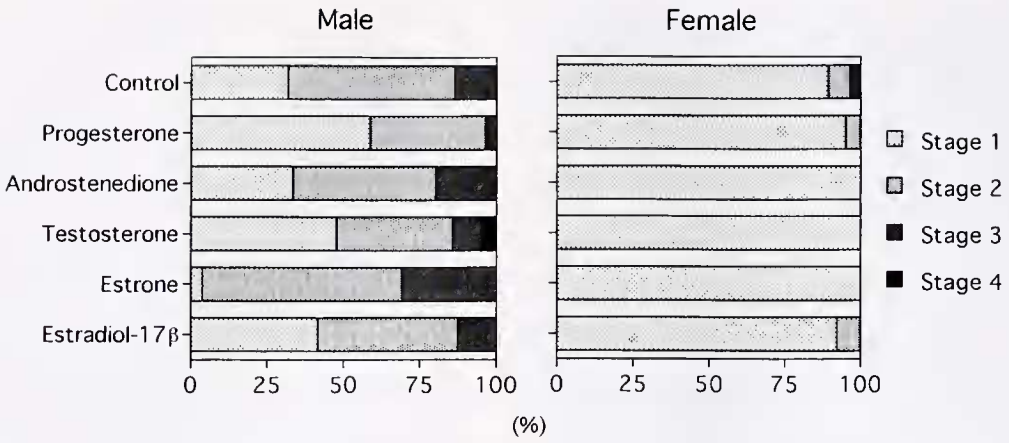

Figure 3. Frequencies of the maturational stages of gonads for male and female *Pseudocentrotus depressus* fed diets containing steroids. The frequencies for male animals in the estrone-treated group are significantly different from those in the control group ($P < 0.05$).

control group. This suggests that spermatogenesis was promoted in the estrone-treated group.

In the females from all groups, most of the gonads were in Stage 1, with no specific difference observed between the steroid-treated and control groups.

Daily food consumption

The mean daily consumption, based on wet matter, for duplicate tanks are shown in Table II. The androstenedione- and the estrone-treated groups showed higher values than the control group, but this difference was not significant.

Discussion

In this study of *P. depressus,* the responses of males and females to orally administered steroids was markedly different. In males, the mean GIs in the androstenedione- and the estrone-treated groups were significantly elevated compared to that in the control group, suggesting that androstenedione and estrone promoted gonadal growth. In these two groups, daily food consumption was higher than in the control group. The mobilization of nutrients from the food into the testes may have been enhanced in these steroid-fed groups.

Unlike males, female animals were not affected by the steroids tested in this study. Unuma *et al.* (1996b) cultured *P. depressus* for one year, from an age of 8 months to 20 months, and during this period most of the females remained immature throughout the annual reproductive cycle, but most of the males commenced gametogenesis. Yearling female red sea urchins thus probably cannot carry out gametogenesis. We think this is the main reason that the female animals did not respond to the steroids in this study.

In both sexes of the sea urchin, the nutritive phagocytes in the gonad are the main site for storage of nutrients required for gametogenesis (Walker, 1982). Nutrients derived from ingested food are assimilated into storage in the nutritive phagocytes before and also during gametogenesis (Takashima, 1976). We suppose that, in this study, androstenedione and estrone promoted the accumulation of nutrients by the nutritive phagocytes. But as gametogenesis progresses, the nutritive phagocytes shrink and lose their nutrient reserves. Therefore, it is difficult to compare the total amount of nutrients accumulated by the nutritive phagocytes of animals that are in different gonadal stages.

Sea urchins are unlike other oviparous animals in that yolk protein is not female specific. The yolk protein accumulates in the nutritive phagocytes as a nutrient source for gametogenesis, not only in females (Ozaki *et al.,* 1986), but also in males (Unuma *et al.,* 1998). Shyu *et al.* (1987) identified, in the sea urchin *Strongylocentrotus purpuratus,* a DNA sequence closely resembling the estrogen-responsive element of vertebrates near the gene of the yolk protein precursor, called vitellogenin. This suggests that vitelloge-

Table II

Daily consumption by Pseudocentrotus depressus *of diets containing steroids*

Diet	Daily consumption (%)[1]
Control	1.985 ± 0.043
Progesterone	1.987 ± 0.002
Androstenedione	2.188 ± 0.073
Testosterone	2.098 ± 0.139
Estrone	2.164 ± 0.026
Estradiol-17β	1.991 ± 0.047

[1] Calculated as 100 × food intake/[(initial body weight + final body weight)/2] × rearing period (days). Values represent the mean ± SE of duplicate tanks. None of these values in steroidal groups is significantly different from that in the control group.

nin synthesis may be controlled by steroids in sea urchins, as it is controlled in oviparous vertebrates by estrogens (Wallace, 1985), and in insects by ecdysteroids (Hagedorn, 1985). In this study, the accumulation of nutrients into the nutritive phagocytes may have been enhanced by the steroids through the synthesis of vitellogenin.

In sea stars of both sexes, estrogens may promote biosynthesis of protein in the pyloric caeca and its subsequent mobilization into the gonads (Schoenmakers and Dieleman, 1981; Voogt and Dieleman, 1984; Voogt *et al.*, 1985; Xu and Barker, 1990). Takahashi (1982a) reported that daily injections of androstenedione and estrone over a 16-day period induced gonadal growth in female *Asterina pectinifera*, whereas progesterone, testosterone, and estradiol-17β did not. Takahashi's results are similar to our observations for male *P. depressus*. Takahashi supposed that androstenedione was metabolized to estrone, which then affected the mobilization of proteins into the ovaries. However, little information is available on the biosynthesis of estrogen from androgens in echinoderms (Hines *et al.*, 1992a). Whether androstenedione itself has the potential to promote gonadal growth in echinoderms must be determined in future studies.

The relationship between steroids and gametogenesis in sea stars has been the concern of several reports. Estrone injections over a 5-day period increased the number of male germ cells in *A. pectinifera* (Takahashi, 1982b). Increases in estrone levels in the testes were observed at the onset of testicular growth in *Asterias rubens* (Voogt and Dieleman, 1984) and in *Sclerasterias mollis* (Xu, 1991). Transient increases in estradiol-17β levels in the testes occurred coincidently with mitotic proliferation of spermatogonia in *Asterias vulgaris* (Hines *et al.*, 1992b). In *A. vulgaris*, after a pretreatment with progesterone, estradiol-17β stimulated spermatogonial mitosis *in vitro* (Marsh and Walker, 1995). These reports suggest that, in sea stars, estrogens are responsible for initiating spermatogenesis through the stimulation of spermatogonial proliferation. In the current feeding experiment, spermatogenesis was promoted in the estrone-treated group. This result suggests that, in sea urchins, estrone is important for the initiation of spermatogenesis, as reported in sea stars. Our conclusion in this study is that androstenedione, estrone, and possibly their derivatives, are probably involved in the reproduction of sea urchins through the control of gonadal growth and gametogenesis.

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